IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

ATTY.'S DOCKET: GROSS32

In re Application of:	)	Art Unit: 1643
	)	
Gideon Gross et al.	)	Examiner: Duffy, Bradley
	)	
Appln. No.: 10/517,784	)	Washington, D.C.
	)	
Date Filed: December 13, 2004	)	Confirmation No. 4624
	)	
For: MEMBRANE-ANCHORED β2 MICROGLOBULIN ...	)	

DECLARATION OF GIDEON GROSS UNDER 37 CFR §1.132

Honorable Commissioner for Patents
U.S. Patent and Trademark Office
Customer Service Window
Randolph Building, Mail Stop Amendment
401 Dulany Street
Alexandria, VA 22314

Sir:

I, the undersigned Gideon Gross, hereby declare and state as follows.

I am a researcher at the Migal Galilee Technology Center, Kiryat-Shmona, Israel. My Curriculum Vitae, including a list of publications, is submitted herewith as Exhibit 1.

Certain experimentation related to the invention described in the above-identified application has been conducted in my laboratory at the Migal Galilee Technology Center. This experimentation is described in the manuscript attached hereto as Exhibit 2.

I either conducted or supervised all of the experimentation described in the manuscript of Exhibit 2. I hereby state, from my own first-hand knowledge, that all of the statements therein are true and the results described therein are true and accurate.

I hereby further declare that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

February 26th 2008 — Gideon Gross
Date

Gideon Gross

Short CV

A. Academic Background

Date (from-to)	Institute	Degree	Area of specialization
1980-1984	Open University, Tel Aviv	B.A.	Life Sciences
1984-1986	Weizmann Institute of Science, Rehovot	M.Sc.	Immunogenetics
1987-1990	Weizmann Institute of Science, Rehovot	Ph.D.	Immunology

B. Previous Employment

Date (from-to)	Institute	Title	Research area
2005-	Tel Hai Academic College	Head, Biotechnology Program	
1995-present	MIGAL	Head of Laboratory	Immunology
1994-present	Tel Hai Academic College	Lecturer, Senior Lecturer	
8-10.2003	Stanford University Medical School, CA, USA	Research Associate (Dr. Ronald Levy)	Tumor immunotherapy
1993-1995	MIGAL	Research Associate	Vaccine Development
1991-1993	Lab. of Mol. Biol. MRC, Cambridge, UK	Post-doctoral fellow (Dr. MS. Neuberger)	Immunogenetics
1991	National Cancer Institute, NIH, MD, USA	Research Fellow (Dr. SA Rosenberg)	Tumor Immunotherapy

List of Publications

1. Gross, G. & Margalit, A. (2007). Targeting tumor-associated antigens to the MHC class I presentation pathway. *Endocrine, Metabolic & Immune Disorders - Drug Targets* 7, 99-109.
2. Margalit, A., Migalovich Sheikhet, H., Carmi, Y., Berko, D., Tzchoval, E., Eisenbach, L. & Gross, G. (2006). Induction of anti-tumor immunity by CTL epitopes genetically linked to membrane- anchored β_2 microglobulin. *J. Immunol.* 176, 217-224.
3. Meyuhas R., Noy, H., Montefiori D., Denisova G., Gershoni J. & Gross G. (2005). HIV neutralization by chimeric CD4-CG10 polypeptides fused to human IgG1. *Mol. Immunol.* 42, 1099-1109.
4. Berko, D., Carmi, Y., Cafri, G., Ben-Zaken, S., Migalovich Sheikhet, H., Tzehoval, E., Eisenbach, L., Margalit, A. & Gross, G. (2005). Membrane-anchored β_2 microglobulin markedly stabilizes a highly receptive state of MHC class I molecules. *J. Immunol.* 174, 2116-2123.
5. Enshell-Seijffers, D., Denisov, D., Groisman, B., Smeliyanski, L., Meyuhas, R., Gross, G., Denisova, G. & Gershoni, J. (2003). The mapping and reconstitution of a conformational discontinuous epitope of HIV-1. *J. Mol. Biol.* 334, 87-101.
6. Schlesinger, J., Arama, D., Noy, H., Dagash, M, Belinki, P. & Gross, G. (2003). In-cell generation of antibody single chain Fv transcripts by targeted RNA trans-splicing. *J. Immunol. Methods* 282, 175-86.
7. Pitcovski, J., Gutter, B., Gallili, G, Goldway M., Perelman, B., Gross, G., Simha Krispel, S., Barbakov, M. & and Michael, M. (2003). Development and large-scale use of recombinant VP2 vaccine for the prevention of infectious bursal disease of chickens. *Vaccine* 21, 4736-43.
8. Margalit, A., Fishman, S., Berko, D., Engberg, J., and Gross, G. (2003). Chimeric beta2 microglobulin/CD3zeta polypeptides expressed in T cells convert MHC class I peptide ligands

into T cell activation receptors: a potential tool for specific targeting of pathogenic CD8(+) T cells. *Int. Immunol.* 15:1379-1387.

9. Wagner, S. D., Gross, G., Cook, G. P., Davies, S. L. & Neuberger, M. S. (1996). Antibody expression from a core human IgH locus reconstructed in transgenic mice from bacteriophage P1 clones. *Genomics* 35, 405-414.
10. Eshhar, Z., Bach, N., Fitzer-Attas, C. J., Gross, G., Lustgarten, J., Waks, T. & Schindler, D. G. (1996). The T-body approach: potential for cancer immunotherapy. *Springer Sem. Immunopathol.* 18, 199-209.
11. Gross, G., Levy, S., Levy, R., Waks, T. & Eshhar, Z. (1995). Chimaeric T-cell receptors specific to a B-lymphoma idiotype: a model for tumour immunotherapy. *Biochem. Soc. Trans.* 23, 1079-1082.
12. Eshhar, Z., Gross, G., Waks, T., Lustgarten, J., Bach, N., Ratner, A., Treizman, J. & Schindler, D. G. (1995). T-bodies: chimeric T-cell receptors with antibody-type specificity. *Methods: A Companion to Methods in Enzymology* 8, 132-144.
13. Gorochov, G., Gross, G., Waks, T. & Eshhar, Z. (1993). Anti-leucocyte function-associated antigen-1 antibodies inhibit T cell activation following low-avidity and adhesion-independent interactions. *Immunology* 79, 548-555.
14. Hwu, P., Shafer, G. E., Treisman, J., Schindler, D. G., Gross, G., Cowherd, R., Rosenberg, S. A. & Eshhar, Z. (1993). Lysis of ovarian cancer cells by human lymphocytes redirected with a chimeric gene composed of an antibody variable region and the Fc receptor gamma chain. *J. Exp. Med.* 178, 361-366.
15. Eshhar, Z., Gross G. & Treisman J. (1993). Modifying the specificity of T cells using chimeric Ig/TCR genes. *Tumour Immunobiology: A Practical Approach*, (Eds) G. Gallagher, R. C. Rees & C. W. Reynolds, Oxford University Press, pp 345-368.
16. Eshhar, Z., Waks, T., Gross, G. & Schindler, D. G. (1993). Specific activation and targeting of cytotoxic lymphocytes through chimeric single chains consisting of antibody-binding domains and the gamma or zeta subunits of the immunoglobulin and T cell receptors. *Proc. Natl. Acad. Sci. USA* 90, 720-724.
17. Gross, G. & Eshhar, Z. (1992). Endowing T cells with antibody specificity using chimeric T cell receptors. *FASEB J.* 6, 3370-3378.
18. Gorochov, G., Lustgarten, J., Waks, T., Gross, G. & Eshhar, Z. (1992). Functional assembly of chimeric T cell receptor chains. *Int. J. Cancer*, Suppl 7, 53-57.
19. Gorochov, G., Gross, G., Waks, T. & Eshhar, Z. (1990). Expression of chimeric antibody/T cell receptor genes as functional receptor molecules in human and mouse T cells. *Cellular Immunity and the Immunotherapy of Cancer*, UCLA Symposia on Molecular and Cellular Biology, New Series, (Eds) M. T. Lotz & O. J. Finn, Wiley-Liss Inc. New York, N.Y. pp 97-101.
20. Eshhar, Z. & Gross. G. (1990). Chimeric T cell receptor which incorporates an anti-tumour specificity of a monoclonal antibody with the cytolytic activity of T cells: a model system for immunotherapeutical approach. *Br. J. Cancer* 62, Suppl. X. 27-29.
21. Gross, G., Waks, T. & Eshhar, Z. (1989). Functional expression of a chimeric T cell receptor with an antibody binding site. *Cellular Basis of Immune Modulation*, Alan R. Liss, Inc., 97-101.
22. Gross, G., Waks, T. & Eshhar, Z. (1989). Expression of immunoglobulin-T cell receptor chimeric molecules as functional receptors with antibody-type specificity. *Proc. Natl. Acad. Sci. USA* 86, 10024-10028.
23. Gross, G., Gorochov, G., Waks, T. & Eshhar, Z. (1989). Generation of effector T cells expressing chimeric T cell receptor with antibody-type specificity. *Transpl. Proc.* 21 (1), 127-130.
24. Luria, S., Gross, G., Horowitz, M. & Givol D. (1987). Promoter and enhancer elements in the rearranged alpha chain of the human T cell receptor. *EMBO J.* 6 (11), 3307-3312.

Materials and Methods

(i) Vectors and expression plasmids. Chimeric β_2m genes were cloned into the mammalian expression vectors pBJ1-Neo or pCI-Neo (Promega, Madison, WI). An XbaI/BamHI stretch coding for mouse β_2m (mβ_2m) leader peptide, the H-2K^b-binding antigenic peptide and the N-terminal part of the linker peptide was constructed with the forward primer 5'GCG TCT AGA GCT TCA GTC GTC AGC ATG GCT CGC 3' and the reverse primer 5'CGC GGA TCC GCC ACC TCC CAG TTT TTC AAA GTT GAT TAT ACT AGC ATA CAA GCC GGT CAG 3' for OVA$_{257-264}$ (SIINFEKL), 5'CGC GGA TCC GCC ACC TCC GAG CCA CAC AAA AAA GTC ATA CAC AGC ATA CAA GCC GGT CAG 3' for TRP-2$_{181-188}$ (VYDFFVWL), or 5' CGC GGA TCC GCC ACC TCC CGG CTG GGC TGT GTT ACA CTC AAA AGC ATA CAA GCC GGT CAG 3' (SEQ ID NO: 80) for MUT1 (FEQNTAQP). Cloning of a BamHI/XhoI fragment encoding mature human β_2m (hβ_2m) with the C-terminal part of the linker peptide and the N-terminal part of the bridge was described. An analogous stretch containing the mature mβ_2m was cloned by RT-PCR using the forward primer 5' GGC GGA TCC GGA GGT GGT TCT GGT GGA GGT TCG ATC CAG AAA ACC CCT CAA 3' (SEQ ID NO: 82) and the reverse primer 5' AAG ACC GTC TAC TGG GAT CGA GAC ATG CTG AGA TGG GAG CCC 3'. The template for mβ_2m gene segments was mRNA from the MD45 T cell hybridoma (H-2$^{k/d}$) and the gene product encodes Asp at the polymorphic position 85. The production of an XhoI/NotI fragment encoding the peptide bridge and the transmembrane and cytoplasmic portion of H-2K^b was described elsewhere. All PCR products were subcloned and their DNA sequence verified. The complete genes were assembled via a single step insertion of the three corresponding fragments into the multiple cloning site of either vector.

(ii) Mice and cell lines. Eight-12-week old C57BL/6 (B6) mice were purchased from Jackson Laboratory (Bar Harbor, ME) and bred at the Weizmann Institute of Science (WIS, Rehovot, Israel) facilities. Animals were maintained and treated according to the WIS animal facility and National Institutes of Health (NIH) guidelines.

(i) Cells. RMA-S is a mutant cell line derived from the C57BL/6 lymphoma RMA (H-2^b), which has defects in peptide presentation by class I MHC molecules due to loss of functional expression of the TAP component TAP-2. These cells can be loaded exogenously with high levels of MHC class I compatible peptides. RMA/OVA and RMA-

S/OVA are clones of these two cells transfected with the full-length chicken ovalbumin gene. MO5 is a chicken ovalbumin-transfected variant of the B16 melanoma, a spontaneously-arising melanoma of C57BL/6 origin. MO5 cells were maintained in DMEM supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 1% sodium pyruvate, 1% non-essential amino acids, combined antibiotics and 500 μg/ml G-418 (Life Technologies, Gaithersburg, MD).

RMA-S cells were transfected with the four dcβ2m-encoding plasmids (see below), and G418-resistant clones were expanded and screened by FACS for expression of the introduced genes. Four clones were selected and expanded: Y314- 7(mOVA), Y317-2(hOVA), Y316-8(mTRP), and Y318- 10(hTRP).

(iii) Peptides. $OVA_{257-264}$ and $TRP\text{-}2_{181-188}$ were synthesized by Dr. M. Fridkin, W.I.S.

(iv) DNA transfection. RMA-S (0.8 ml) at $4x10^6$ cells/ml were mixed in 4 mm sterile electroporation cuvette (ECU-104, EquiBio, Ashford, UK) with 20 μg linearized plasmid DNA. Transfection was performed with an Easyject Plus electroporation unit (EquiBio) at 250V, 750 μF. Cells were resuspended in fresh medium and cultured for 24-48 hours in 96-well plates prior to addition of G418 to a final concentration of 1 mg/ml. Resistant clones were expanded in 24-well plates and screened by flow cytometry for expression of $h\beta_2m$ or increase in expression of surface $H\text{-}2K^b$.

(v) Tumor immunotherapy. Ten mice in each experimental group were inoculated s.c. in the upper back with $1x10^5$ MO5 cells/mouse. Local tumor diameter was measured with calipers. Starting 8 days later, when the tumor reached 3-4 mm in diameter, mice were immunized i.p. four times at 7-day intervals with $2x10^6$ irradiated transfectants or control cells pre-loaded with peptide at 50 μg/ml. Tumor diameter and survival were recorded.

(vi) Statistical analysis. Statistical differences in tumor sizes between groups of mice was determined by one-way ANOVA. Significance of survival plots was done with Kaplan-Meier survival platform. For both analyses we used the JMP statistics software (SAS Institute, Cary, NC).

Induction of protective antitumor immunity by transfectants We assembled genetic constructs comprising dcβ2m, attached to the cell membrane via the H-2Kb appendage. As a tumor model, we chose MO5, which expresses both chicken OVA as a xenoantigen,

providing the immunodominant H-2K^b-binding OVA$_{257-264}$ peptide and TRP-2, a self-melanocyte differentiation Ag, harboring the poorly immunogenic peptide TRP-2$_{181-188}$, which binds H-2K^b with low affinity.

We tested the ability of dcβ2m cell-based vaccines to provide protection against melanoma. To this end, we immunized B6 mice three times with Y317-2(hOVA) or Y318-10(hTRP) and with RMA-S cells preloaded with OVA257–264 or TRP-2181–188 peptide for comparison or with the 3LL Lewis lung carcinoma-associated MUT1 peptide as a negative control. Twelve days after the last inoculation, mice were challenged with 1 x 10^5 MO5 melanoma cells, and tumor size, along with animal survival, were recorded. As shown in **Fig. 4A**, expression of the TRP-2181–188-bearing construct by Y318-10(hTRP) cells had a significant protective effect, compared with TRP-2181–188-saturated RMA-S cells ($p < 0.0001$). Six of eight mice that received Y318-10(hTRP) remained tumor-free 7 wk after tumor challenge, compared with two of seven mice immunized with TRP-2181–188-loaded cells ($p = 0.04$) and only one of eight following immunization with MUT1-loaded cells (**Fig. 4B**). All mice (eight of eight) immunized with Y317- 2(hOVA) remained tumor-free during the same period, compared with six of eight mice immunized with RMA-S cells saturated with OVA257–264. However, in light of the relative efficacy of OVA257– 264-pulsed cells, no statistical significance could be derived for the latter set of data.

Immunotherapy of tumors. To evaluate immunotherapy of melanoma, we performed an experiment of tumor growth inhibition. B6 mice were challenged with 1x10^5 MO5 cells each. Starting eight days later, mice were subjected to an immunization regimen with either irradiated Y317-2(hOVA), parental RMA-S cells pulsed with OVA$_{257-264}$, or with PBS only as control. As evident from **Fig. 5A**, tumor growth was significantly delayed in mice vaccinated with Y317-2(hOVA) compared to the peptide-loaded cells ($p<0.0001$). This therapeutic effect was also evident from the survival graph (**Fig. 5B**). Of 10 mice vaccinated with Y317-2(hOVA), 8 were still alive 7 weeks after tumor challenge, compared with 3 of 10 of mice vaccinated with RMA-S cells loaded with the peptide ($p<0.0001$) and 0 of 10 of non-immunized mice. In contrast, immunization with Y318-10(hTRP) and TRP-2$_{181-188}$-loaded RMA-S cells under the same experimental conditions failed to yield any significant MO5.

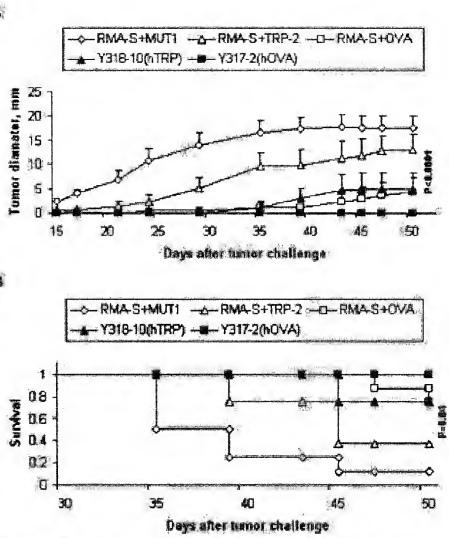

FIGURE 4. Comparative analysis of tumor protection conferred by RMA-S transfectants and peptide-loaded RMA-S cells. Groups of 10 8- to 12-wk-old female B6 mice were immunized three times i.p. with the indicated irradiated cells. Twelve days after the last immunization, MO5 tumor cells were administered s.c. *A*, Inhibition of tumor growth. Local tumor dimensions were measured with calipers. The average of tumor diameters (in millimeters) in the course of 50 days is presented. The results are presented as mean + SEM. p value is shown for the group immunized with Y318-10(hTRP) against the group immunized with TRP-$2_{181-188}$-loaded RMA-S. *B*, Survival of immunized mice. Mice from the same experiment were monitored daily and were sacrificed when moribund, which corresponded to a tumor diameter of ~20 mm. Fraction of surviving mice in each group is presented. p value is shown for the same groups as in *A*.

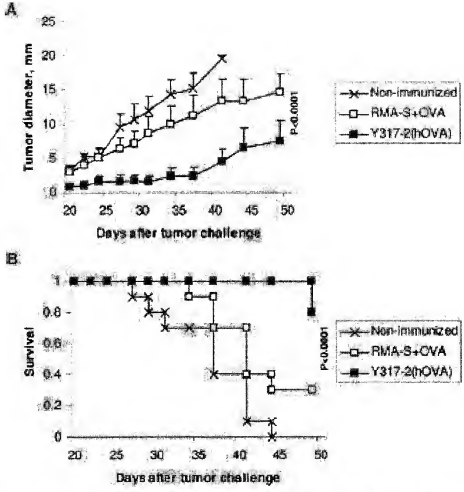

FIGURE 5. Inhibition of tumor growth. MO5 tumor cells (1×10^5/mouse) were injected s.c. to female B6 mice (8–12 wk old). Eight days later, when tumor diameter reached 3–4 mm, mice were divided to groups of 10 and were immunized i.p. four times at 7-day intervals (days 8, 15, 22, and 29) with irradiated Y317-2(hOVA) or RMA-S cells loaded with 50 μg/ml $OVA_{257-264}$ or with PBS only (nonimmunized). *A*, Tumor progression. Local tumor dimensions were measured with calipers. The average of tumor diameters (in millimeters) in the course of 50 days is presented. *B*, Survival of immunized mice. Mice from the same experiment were monitored daily and were sacrificed when moribund, which corresponded to a tumor diameter of ~20 mm. Fraction of surviving mice in each group is presented. Data are representative of two independent experiments with similar results. The results are presented as mean + SEM. Both *A* and *B* present *p* values calculated for the two groups of immunized mice.